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Effect of proteins on population size of *Entodinium longinucleatum* Dogiel in cultures

Wpływ białek na liczebność *Entodinium longinucleatum* Dogiel w kulturach

The investigations repeatedly undertaken with the aim to establish the medium required by the representatives of *Entodiniomorpha* were a success not on all occasions due to considerable obstacles arising in reproducing the conditions existing in the rumen. The culture of these ciliates outside the host's organism presents favourable conditions for investigating the physiology of these *Infusoria*. The problem is intricated by the fact of a considerable specificity degree of this protozoan group and of its dependence on the bacterial flora of the rumen.

Knoth 1928 was the first to study the methods for in vitro cultures on the grounds of an analysis of the conditions existing in the rumen. In his experiments, he applied rumen juice with ground hay, an established pH of nutrient medium, and aerating with CO_2 and CH_4 mixture. The longest duration period of the *Entodinium minimum* culture was 5 days.

The investigations by Margolin 1930 and those by Hungate 1942, 1943 were chiefly aimed at establishing the part the infusorians played in the process of utilization of cellulose; it ought to be put to the latter author's credit that the fact of some infusorian species possibly feeding on plants was proved in part. So for instance, *Eudiplodinium neglectum* developed for 22 months on a nutrient medium of inorganic salts ($NaHCO_3$, KH_2PO_4, $MgSO_4$, $CaCl_3$, NaCl) with grass and cellulose added. The monocultures obtained and the preparation of the extract enabled Hungate to establish cellulase in *Eudiplodinium neglectum, Diplodinium maggii, Diplodinium multivesiculatum,* and *Diplodinium denticulatum,* and the absence of this enzyme in *Entodinium caudatum.*

Westphal 1935 studied the problem of the ability of the infusoria to utilize other carbohydrates. He cultivated the protozoans on the fresh rumen juice with some rice starch and urea added. The species of the genera *Anoplodinium, Eudiplodinium, Epidinium* and *Isotricha* survived only for 10 days under these conditions, and *Entodinium* alone continued to live in the culture lasting for 36 days; its population attained some 70 000 specimens per 1 cc.

The problem was also investigated by Ciemochowska-Mazik 1956 who studied the possible utilization of various kinds of starch by a particular

species of infusorians. The authoress mentioned above cultivated *Entodinium longinucleatum* on sterile rumen juice with addition of ground solid parts of rumen, and starch: of barley, rye, wheat, soy, rice and maize. The best results within a 6-day period of culture were obtained on barley starch (40500/s cc.) and on soy starch (41 500/1 cc.).

Coleman 1958 who partly changed both the nutrient medium (the solution of K_2HPO_4, KH_2PO_4, NaCl, $MgSO_4$, $CaCl_3$, CH_3COONa, Na_2S with grass and rice starch), and the methods of culture worked out by Hungate, obtained good results of experiments. After four months only the following species were still alive in the culture: *Entodinium longinucleatum*, *E. caudatum*, *E. simplex* (the two latter being considered by Poljanskij and Strelkov as synonyms), and *Eudiplodinium medium* (according to the author *Metadinium medium*).

Willing 1930, in looking for new methodic solution, applied a series of chemical compounds (sodium phosphate, calcium carbonate, magnesium oxide, glycerol) rice meal, ground wheat, alfalfa and casein; their addition to the rumen juice aimed at securing proper conditions for the development of *Infusoria*. However, these cultures failed to justify the expectations; the longest period, in the case of addition of casein, was of 120 hours. Willing who gave animal protein to the infusorians was the first to try to ascertain which kind of protein was absorbed by the rumen infusorians.

Ferber 1928, and Mowry et Becker 1930 were the other investigators who worked at the definition of the origin of the protein in infusorians, mainly in carrying out their experiments on domestic animals. Westphal 1935, on the margin of his study on the carbohydrates metabolism points to two different protein sources available to *Infusoria*: the bacterial and the vegetable protein. He also observed stained bacteria, absorbed by the infusorians, which subsequently were digested. Sufficiently reliable evidence of the vegetable protein being possibly digested or the corroboration of the infusorians development depending on the presence of definite species of bacteria is essential in solving the problem of the part the protozoans play in the nutrition of the ruminants.

The present study aims at establishing the effect of the particular fractions of wheat protein on the population size increment of a definite species, namely *Entodinium longinucleatum* Dogiel. This species was selected due to its morphological characters being relatively constant (e. g. size and location of the macronucleus or that of the caudal lobe), and due to this infusorians occurring abundantly in the rumen.

The present authoress wishes to express her most sincere gratitude to Professor Dr. Marian Rybicki for both his choosing the theme, and the kind assistance in preparing the present work.

Methods

The preliminary work consisted in preparing the nutritive medium comprising fractions of protein obtained from wheat flour, barley starch and serradilla hay, collected at blossoming period. The protein was isolated from wheat flour after Hawk, Oser et Summerson 1937 (p. 177). The

above mentioned solid components of nutrient medium were ground in a porcelain mortar and sifted through sacks of mill gauze. The liquid component consisted of filtered juice of the cattle rumen, two times sterilized prior to each transfer.

The methods of culture, in the main, corresponded to the pattern worked out by Ciemochowska-Mazik 1956; however, some important details deserve particular attention. Every 24 hours, 10 ml. infusorian-containing juice were transferred onto fresh nutrient medium including the proper solid components in another 10 ml. rumen juice. The magnitude of the amount of the material to be transferred was established after several experiments.

The established average duration of CO_2 bubbling showed insufficient in the case of the cultures described here. Due to the specific and strenuous fermentation process occurring after protein was added, a new gas saturation of the culture was necessary. This used to be done not earlier than 7 hours following transfer onto fresh nutrient medium. The introduction of the above modification brought about improvement in the conditions of culture.

A pure culture of *Entodinium longinucleatum* was obtained by the dilution methods 14 days following the transfer of the infusorians collected from the cattle rumen. Nutrient medium with starch added showed the most appropriate one and, by means of frequently repeated transfers, very soon a culture was obtained in which one species survived.

The infusorian cultures were grown on nutrient mediums containing three sorts of wheat protein: gluten, gliadin and glutein.

Four series of experiments were carried out on each of these proteins, the following food stuff composition being utilized:

a) 10 mg. protein (gluten and glutenin, resp.)
   30 mg. barley starch — for a period of 21 days

b) 20 mg. protein (gluten and gliadin, resp.)
   30 mg. barley starch — for a period of 21 days

c) 20 mg. protein (gluten, gliadin and glutenin, resp.)
   30 mg. barley starch
   20 mg. serradilla — for a period of 21 days

d) 20 mg. protein (gluten and gliadin, resp.)
   30 mg. barley starch
   20 mg. serradilla in two parallel cultures — for periods of 112 resp. 105 days.

The initial infusorian culture in ali experiments was one grown on gluten (20 mg. gluten + 30 mg. barley starch).

The quantity of organisms initially introduced, and the magnitude of the first sample transferred are important factors affecting the population size of the infusorians during the 21 day periods.

Important disturbances in the fermentation process, showing in the medium odour turning sour, are caused by an insignificant excess in the protozoan population size resulting from considerable dilution with fresh nutrient medium. Observations have shown that these very harmful changes may

be avoided, at least partly, when a new series is transferred onto a definite protein. The most favorable conditions were established when the amounts both of the fresh medium and of the infusorian-containing liquid were equal. The collecting of the infusorians from one culture in as short period of time as feasible was another important factor to be born in mind.

The initial number of infusorians in the various series was exactly defined and fluctuated about 1000 specimens per 1 cc.

The Fuchs-Rosenthal chamber was utilized for the daily computations as were carried out for the particular culture. The portion of the sample as remained after transferring, some 10 ml. liquid which often contained very mobile infusorians, was cooled in a refrigerator during a period of 40 minutes at least. The liquid was thoroughly stirred after which two chambers were filled simultaneously and the number of infusorians on the whole surface was counted. Twenty measurements were carried out to obtain the daily mean number of infusorians in one culture. The number of infusorians in one cc. was computed (taking into account the capacity of the chamber). The population sizes of the infusorians in cultures on one particular medium varying very considerably, a mean of the measurements of 5 average parallel cultures was taken.

Experiments

1. Cultures with gluten added.

A series of cultures with reduced amount of protein (10 mg.) was started by transferring infusorians in the proportion of 3000 or 4000/1 cc. onto a culture with a double gluten dose (20 mg.) containing 6000 to 7000 infusorians per 1 cc., and 1000 to 2000 specimens per 1 cc. were transferred onto serradilla enriched medium. The initial numbers of infusorians on the two remaining proteins (gliadin and glutenin) were constant in all of the subsequent series.

The results of the counts of infusorians in three gluten series are given in Table I; they are also illustrated by the successive curves in Diagram I. The curve 1 shows the average infusorian numbers in three cultures grown on nutrient medium with 10 mg protein added. Four periods of infusorian population increase, lasting for 4 or 5 days each, occurred during the 21-day culture. The maximum infusorian level during the particular periods is rather similar in all cases. The mean number of infusorians per 1 cc. attained its maximum (12100) on the 7th day of culture. The successive maxima, on the 12th, 15th and 20th day attained 10100 to 11400/1 cc.

In order to ascertain whether the results obtained represented the maximum possible under the given conditions of infusorian population size and whether the amount of protein met the requirements of these organisms, the dose of gluten was doubled (20 mg.). The population size of the infusorians increased by 100 per cent. (Table I) as compared to the first cultures (10 mg.). A more significant increase in the infusorian population size was

observed at the beginning of the culture (2nd day); the average numbers of protozoans remained similar (Table I, series 2) during the following 4 days (12600 to 14500/1 cc.), while the maximum population size was attained on the 9th day. This was the highest concentration degree of protozoans obtained in a culture in vitro on medium consisting of protein and barley starch. A decrease in the population size of the infusorians began on the 10th day of culture; the population decreased very rapidly from 25400/1 cc. to 8400/1 cc. during 4 days; then, after a short period of insignificant fall of the population size, a one-day period (on the 17th day of culture) of increase followed with subsequent fall of the infusorian proportion to 3900/1 cc. (Diagram I, curve 2).

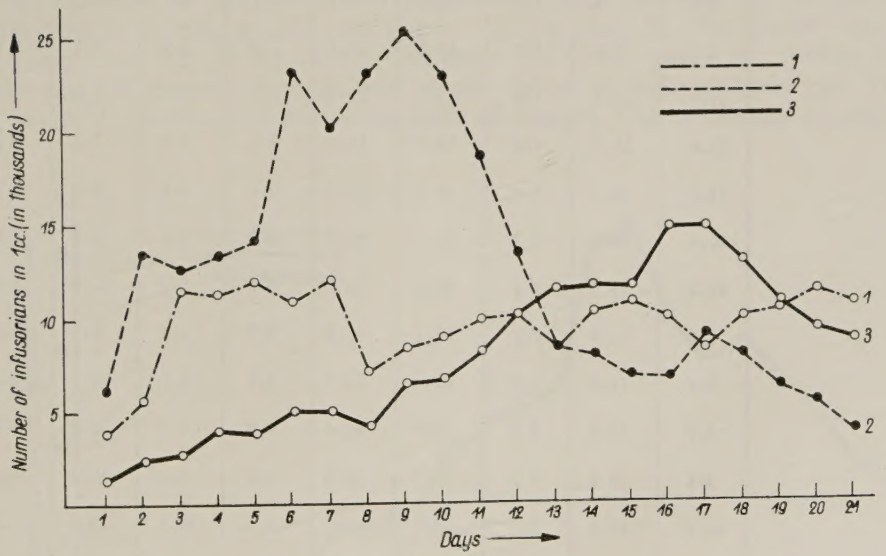

Diagram I. Cultures of *E. longinucleatum* with gluten added
Compound of medium: 1. barley starch — 30 mg., protein — 10 mg., 2. barley starch — 30 mg, protein 20 mg.; 3. barley starch — 30 mg., protein — 20 mg., serradilla — 20 mg.

The analysis of the mean population size of infusorians in four parallel cultures (Table I, series 2) discloses some particular features deserving more attention. An increase in the infusorian population size was observed in all cultures while maximum increase on the 9th day was observed in two cultures while the maximum as was obtained in the remaining cultures was considerably lower and occurred as late as on the 17th and 18th day of the whole experimental period.

A series of experiments on gluten exhibits considerable fluctuations in the number of infusorians (see Table I, columns 5 and 6); these differences were established in all of the experiments. Only those values as were most approached to the means, were utilized in computing the mean values.

Table I
Cultures with gluten added

Number of series	series 1 - 3 cultures			series 2 - 4 cultures			series 3 - 5 cultures		
Period of culture	6.IV. - 3.VI.			9.IV. - 17.VI.			8.IV. - 30.VI.		
Amount of barley starch	30 mg.			30 mg.			30 mg.		
Amount of gluten	10 mg.			20 mg.			20 mg. + serradilla		
No.of infusoria in one cc (in thousands)	Average	Maximal in one of the cultures	Minimal in one of the cultures	Average	Maximal in one of the cultures	Minimal in one of the cultures	Average	Maximal in one of the cultures	Minimal in one of the cultures
	1	2	3	4	5	6	7	8	9
	3.8	3.9	3.6	6.5	6.9	6.1	1.4	1.8	1.0
	5.7	7.9	3.5	13.5	16.3	7.9	2.5	3.1	1.7
	11.5	21.3	3.9	12.6	22.7	7.2	2.7	4.9	1.7
	11.4	15.9	4.0	13.4	19.6	7.3	4.0	7.9	0.7
	11.9	20.2	5.4	14.5	21.7	9.1	3.9	8.6	1.1
	10.9	16.3	5.2	23.3	34.5	12.9	5.1	8.3	1.6
	12.0	19.9	3.7	20.1	31.1	9.1	5.0	11.8	2.2
	7.0	11.3	2.7	23.1	42.0	6.4	4.2	6.3	2.5
	8.4	12.9	1.9	25.4	33.6	3.8	6.5	13.1	2.7
	8.9	12.5	3.6	22.9	40.4	2.8	6.7	12.9	2.9
	9.8	18.6	3.9	18.7	35.0	3.8	8.1	15.0	4.2
	10.1	18.5	3.7	13.4	21.0	4.6	10.1	16.0	5.2
	8.3	16.2	2.8	8.4	16.6	4.4	11.5	16.8	6.6
	10.3	24.8	2.0	8.0	14.2	3.7	11.7	15.9	9.8
	10.8	19.7	2.6	6.9	10.2	4.7	11.6	16.9	4.8
	10.0	17.5	5.1	6.8	8.0	5.7	14.8	30.0	6.9
	8.3	13.2	4.5	9.1	13.3	6.5	14.8	25.8	4.4
	10.0	13.9	7.5	8.1	14.8	3.7	13.1	23.0	5.2
	10.4	13.5	7.6	6.3	8.1	5.3	10.7	16.1	6.1
	11.4	20.5	6.4	5.4	6.9	4.2	9.4	16.7	6.3
	10.8	24.8	3.5	3.9	5.5	2.9	8.8	19.1	4.7

In the third series of experiments (Table I), dried serradilla leaves were added to the nutrient medium alongside the gluten. This addition of ground serradilla improved the conditions of culture. The curve 3, in Diagram I, illustrates the characteristic gradual increase as lasted during a longer period of culture (15 days). The maximum level of infusorian population was established during the last 6 days, in 5 parallel cultures. The largest population size obtained on the 16th and 17th days of culture (14800 infusorians per 1 cc.) somewhat exceeded that attained on 10 mg. gluten in series 1 (12000 specimens per 1 cc., on the 7th dey, see Table I, columns 1 and 7).

2. Cultures with gliadin added.

Mean sizes of the protozoan population were attained in the cultures after adding 10 mg. gliadin (see curve 1 in Diagram II). The initial increase in population size of the infusorians (6300 specimens per 1 cc.) lasting for 4 days was the maximum mean size of population in these cultures (see Table II, series 1). During the following days of culture, the infusorian population

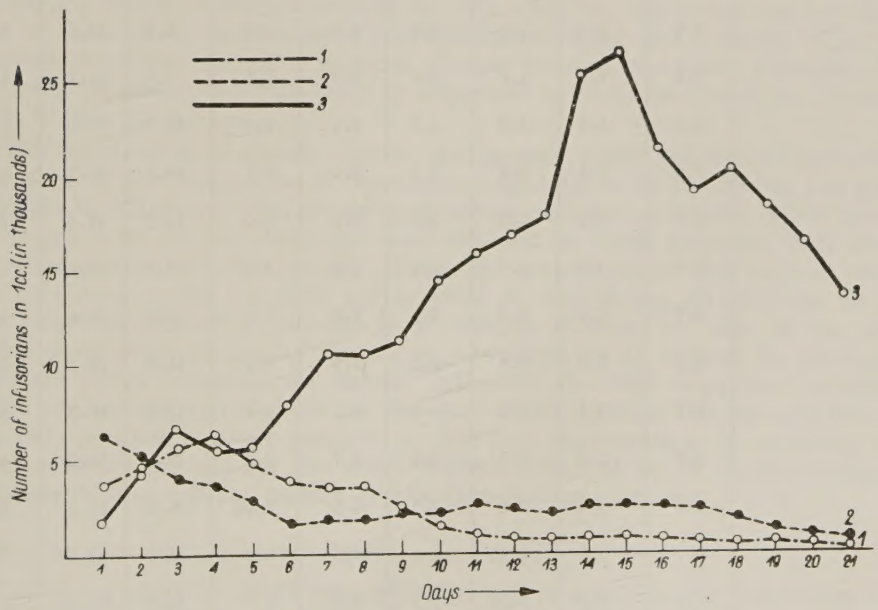

Diagram II. Cultures of *E. longinucleatum* with gliadin added
Compound of medium: 1. barley starch — 30 mg., protein — 10 mg.; 2. barley starch — 30 mg., protein — 20 mg., 3. barley starch — 30 mg., protein — 10 mg., serradilla — 20 mg.

size gradually diminished falling to the level of some 700/1 cc. on the 12th day. The established population size did almost not vary during the subsequent period of culture. Within the last few days, there was less then 100 infusorians per 1 cc. in three of the five parallel cultures (see Table II, column 3). It ought to be stressed that the course of the experiments was very regular

Table II
Cultures with gliadin added

Number of series	series 1 - 5 cultures			series 2 - 5 cultures			series 3 - 5 cultures		
Period of culture	31.V. - 9.VII.			29.VII. - 25.VIII.			1.VI. - 21.VII.		
Amount of barley starch	30 mg.			30 mg.			30 mg.		
Amount of gliadin	10 mg.			20 mg.			20 mg. + serradilla		
No.of infusoria in one cc (in thousands)	Average	Maximal	Minimal	Average	Maximal	Minimal	Average	Maximal	Minimal
		in one of the cultures			in one of the cultures			in one of the cultures	
	1	2	3	4	5	6	7	8	9
	3.6	3.9	3.3	6.2	6.5	6.1	1.8	2.0	1.5
	4.5	6.6	1.5	5.2	5.5	4.6	4.5	6.2	2.0
	5.6	11.2	2.4	3.9	6.3	1.7	6.8	11.5	2.0
	6.0	9.6	4.6	3.6	5.5	1.1	5.6	10.9	1.4
	4.8	9.1	1.9	2.8	5.0	0.8	5.7	13.6	1.4
	3.8	6.1	1.4	1.6	3.7	0.6	7.9	15.7	1.4
	3.5	6.8	1.2	1.7	5.6	0.5	10.7	22.0	3.2
	3.5	6.6	0.4	1.7	5.0	0.7	10.6	22.3	1.4
	2.3	7.7	0.5	2.0	5.0	1.0	11.3	17.7	2.5
	1.4	3.3	0.5	2.0	2.9	1.2	14.4	29.4	1.3
	0.9	1.9	0.2	2.5	3.8	1.5	15.9	30.4	2.3
	0.7	1.2	0.4	2.2	3.9	0.9	16.8	29.7	2.0
	0.7	1.1	0.1	2.0	4.1	0.4	17.9	46.0	3.4
	0.8	1.9	0.2	2.4	5.7	0.2	25.5	56.2	4.6
	0.8	1.4	0.1	2.5	6.1	0.06	26.6	63.0	2.8
	0.7	1.6	0.03	2.4	4.1	0.0	21.5	51.5	2.2
	0.6	1.2	0.03	2.3	4.3	0	19.3	41.0	0.9
	0.4	1.2	0.02	1.8	4.7	0	20.4	51.1	1.0
	0.6	2.0	0.02	1.1	2.2	0	18.5	32.0	0.5
	0.5	1.6	0.03	1.0	2.2	0	16.5	27.4	0.6
	0.3	1.1	0.02	0.7	1.8	0	13.7	25.0	0.2

in most of the cultures (in four of them), and the infusorian population size was very similar on each given day.

A similar result was obtained in another experiment carried out on a larger amount of gliadin (20 mg., see Table II, series 2). The largest population size of the infusorians did not exceed the limit defined by the curve of the population size increase (1) in the series on 10 mg. protein. The drop in the population size (Diagram II, curve 2), as showed at the moment of the first transfer, continued for six days; then a mean level of some 2000/1 cc. fixed; this rather insignificant level of population remained till the 17th day of the period of experiment falling then as low as 700/1 cc. The infusorians died out as early as the 16th day (Table II, column 6) in one of the 5 cultures grown for this series while in two others, the infusorians totalled 31 to 172 specimens per 1 cc. within the last three days of culture. The course of the two remaining cultures was not identical in character, still, the population size kept on the average level attained in all of the cultures (not above 6500 infusorians per 1 cc., see Table II, column 5).

The results of the experiments show the addition of gliadin to have no favourable effect on the development of the infusorians. The change in fermentation would probably account for this fact for, depending on the duration of the culture, the nutrient medium turned sour and a heavy odour developed, characteristic of a medium altered by decomposition processes; the products of these processes may be supposed to have inhibited the development of the infusorians.

The third series of gliadin culture was grown under addition of serradilla. The data on the mean size of infusorian populations in 5 cultures are given in Table II, column 7. The largest population size ever attained (26000 protozoans per 1 cc. on the average) was obtained in these cultures. This result has been somewhat unexpected taking into consideration the serious obstacles met with in the two first series after 10 and 20 mg. gliadin was added. The effect of serradilla resulted in a uniform increase in size of the infusorian population shown by the curve 3 in Diagram II. It may be concluded from the curve (showing no distinct fall until the 15th day) that serradilla added to the nutritive value of the diet of the infusorians thus favorably affected increase in their population. Further observations on mobility and odour (resembling that of the cattle rumen) after 24 hours suply additional evidence of the normal course of the fermentation process.

3. Cultures with glutenin added.

Following addition of 10 mg. glutenin to the medium, the infusorian cultures in the first series equalled population size of the gliadin cultures in the same series — 6300/1 cc. However, on comparing the two curves (the first ones, see Diagram II and III) they turn out different in character. While the curve of the gliadin culture exhibits a falling tendency, the population size of the infusorians on the glutenin culture reaches the maximum height obtained after 14 days — 6300/1 cc. (see Table III, series 1). It results from a careful analysis of curve 1 that the mean number of the infusorians remains steadily at the level of $\pm$ 3000/1 cc. up to the 12th day of culture (see Table III, column 1). An increase in the population size occurred on the 13th day, the

maximum population size in the individual four cultures varying very considerably (conf. Table III, columns 2 and 3).

The mean sizes of the infusorian population in the cultures with serradilla added were very similar to those obtained on the gliadin culture. Curve 3, in Diagram III, shows a rather steady increasing tendency of the infusorian population size up to the 20th day of culture. This is characteristic of cultures grown on various sorts of protein with serradilla added. The diagram depicts

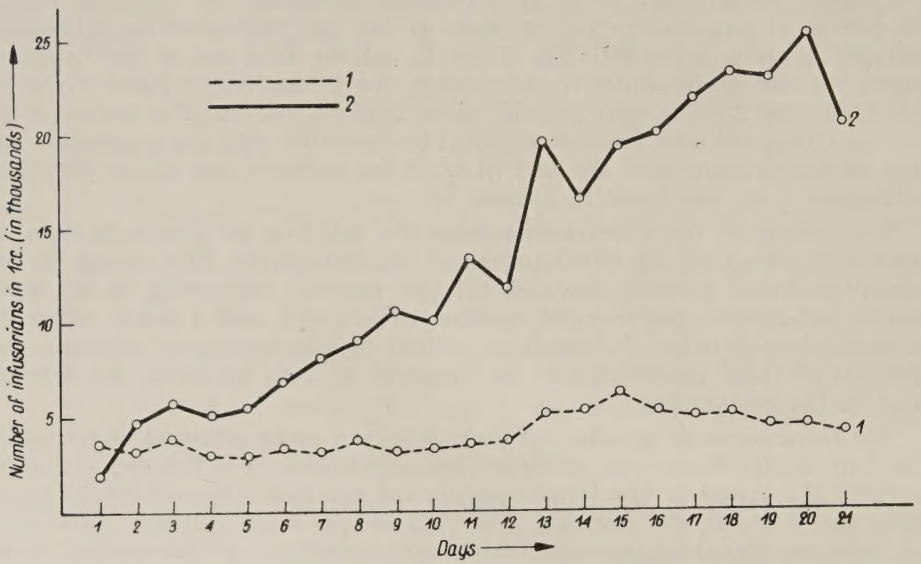

Diagram III. Cultures of *E. longinucleatum* with glutenin added
Compound of medium: 1. barley starch — 30 mg., protein — 10 mg.; 2. barley starch — 30 mg., protein — 10 mg., serradilla — 20 mg.

the data obtained from two cultures in this series for, in the remaining two cultures, the populations of the infusorians attained levels deviating too considerably from the mean value. This uneven level of the population size seems to be demonstrative of changes as occurred under the conditions of cultures in this particular series.

The results obtained in third series, on a medium containing various sorts of protein (gluten, gliadin, glutenin) with addition of serradilla, permit to conclude that the supplement of the ground plant always affects the fermentation processes very positively. In the cause when the infusorians be transferred from the gluten-serradilla medium onto glutenin-serradilla one, even the presence of serradilla does not diminish the differences caused by the sort of protein supplied. The contents of the culture turned slimy and markedly sour within the first three days following transferring from the gluten-serradilla medium; most of the cultures as were planned could be not laid irrespective the through ventilation. Table III contains the counts carried out in the cultures as could be admitted for calculations.

Table III
Cultures with glutenin added

Number of series	series 1 - 4 cultures			series 3 - 2 cultures		
Period of culture	28.VII. - 24.VIII.			23.VIII. - 13.IX.		
Amount of barley starch	30 mg.			30 mg.		
Amount of glutenin	10 mg			20 mg. + serradilla		
No. of infusoria in one cc (in thousands)	Average	Maximal in one of the cultures	Minimal	Average	Maximal in one of the cultures	Minimal
	1	2	3	4	5	6
	3.6	3.7	3.5	1.9	2.0	1.9
	3.2	5.0	1.9	4.7	5.9	3.5
	3.0	7.1	2.2	5.7	8.4	3.0
	3.9	5.4	1.4	5.1	6.1	4.2
	2.9	5.7	1.9	5.5	5.7	5.2
	3.3	7.4	1.6	6.9	-	-
	3.1	6.1	1.9	8.1	8.9	7.4
	3.7	9.0	1.3	9.0	10.9	7.1
	3.1	7.2	0.5	10.6	15.4	5.9
	3.3	6.6	1.5	10.0	13.8	6.3
	3.5	5.8	1.6	13.4	19.4	7.3
	3.8	6.3	1.3	11.8	16.2	7.4
	5.2	9.5	1.9	19.7	26.1	13.3
	5.2	9.9	1.7	16.4	22.5	10.2
	6.3	11.4	2.3	19.4	20.8	17.9
	5.3	7.8	2.5	22.0	23.4	20.6
	5.2	10.3	1.8	23.4	26.2	20.6
	4.5	6.1	2.6	23.2	24.7	21.7
	4.6	8.3	2.7	25.6	30.7	20.5
	4.2	7.2	3.0	20.8	34.5	7.2

4. Long-term cultures.

The gluten culture was controlled from April 7th to July 27th, i. e., for a period of 112 days, till the moment of total extinction of the protozoans. Several stages characteristic of the particular periods of culture may be distinguished in the curve 1 (Diagram IV) showing the numbers of the infusorians. The size of the population attained very high level on two occasions within the initial period lasting for 18 days in April. After a seven-day period of increase (32844/1 cc.) a rapid drop in the number of the infusorians followed which then gave way to a new period of intense increase of the population size resulting in a very high level of population concentration with 41647 protozoans per 1 cc. The second stage of culture was characterized by a decrease of the infusorians, rapid at the beginning, then moderate for a period of 18 days. The lowest level was 750/1 cc. during this period. During the third stage — from May 14th to June 15th — three periods of population increase occurred successively similar in size (8906, 6812 and 6953 specimens per 1 cc. respectively). The decrease in the population size of the protozoans, after the peaks were attained, lasted for 4 days only. The successive, fourth, increase in the population size was relatively important with 16781 infusorians per 1 cc., while the further one, 8 days latter, attained the level of 7281 only. The level was below 900 specimens per 1 cc. within the final stage, lasting for 20 days (July); the protozoans averaged 50 specimens per 1 cc. within the last 4 days of culture.

The gliadin culture was grown for 105 days from June 8th till September 20th. An increase in population size was observed on the third day — 10047/1 cc. — which exactly corresponded to the population density occurring in the 21-day cultures. After this, an 18-day period of decrease followed during which the level of the population dropped to 800/1 cc. on two occasions. Within the following 17 days a very high level of 63000/1 cc. was attained and, after a 2-day decrease, 51116 protozoans were established in 1 cc. The subsequent long-term decrease in the protozoan population size lasted for 17 days. This period was marked by sudden changes with a relatively low average level of 10000/1 cc. The following stage — from August 9th till August 21st — was notable for its very high level held out within further 17 days. After a few variations, a maximum level of 83766/1 cc. was attained which was the highest population size ever attained in a culture in vitro. Then, a fall followed which lasted for 13 days dropping, at the end, to the value of 9844/1 cc. During the last stage, the level rised attaining 30906 specimens in 1 cc.; after this, the level dropped to 6547/1 cc. Taking into consideration the course of experiment carried out so far, the final fall can not be considered indicative of the possible extinction of the culture.

The quantitative results obtained in the cultures reported here suggest the conclusion — corroborated by the study of the 21-day cultures — that adding the particles of serradilla to the nutrient medium created the proper conditions for the infusorian development.

In periodic (21 days) cultures grown on medium containing proteins of wheat, the infusorian population attained the following levels: on 20 mg. of gluten — 25400/1 cc. (the highest level attained); on 10 mg. — only 12100/1 cc.; the result of the 10 mg. glutenin culture was 6300/1 cc., while in

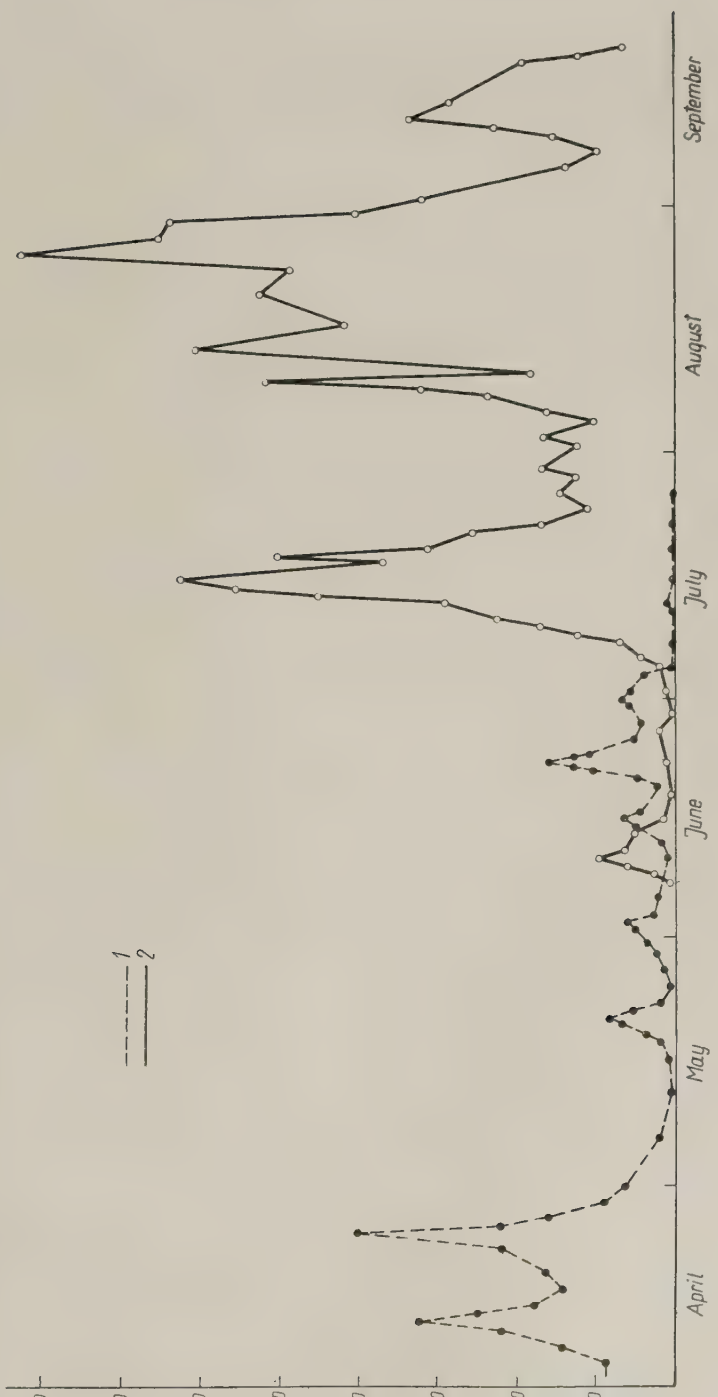

Diagram IV. Cultures of *E. longinucleatum* with gluten or gliadin added

Compound of medium: 1. barley starch — 30 mg., gluten — 20 mg., serradilla — 20 mg. (7.IV — 27.VII);
2. barley starch — 30 mg., gliadin — 20 mg., serradilla — 20 mg. (8.VI — 20.IX)

the case of gliadin the gradual drop in both the 10 mg. and the 20 mg. culture was indicative of unproper medium.

In the cultures with serradilla added the highest level was obtained on gliadin (26600/1 cc.), somewhat similar result was obtained on glutenin (25600/1 cc.), while that attained on gluten was the lowest (14800/1 cc.).

Discussion

On the grounds of the results obtained, *Entodinium longinucleatum* was confirmed to attain different levels of population size in cultures in vitro depending on the sort of the vegetable protein contained in nutrient medium. The diverse composition of the amino-acids contained in the proteins introduced into the medium ought to be regarded as the main reason for this phenomenon.

The problem of the utilization of the vegetable proteins is a very difficult one and the only conclusion from the present paper is the increase in the infusorian populations size in the particular series of experiments. In the cultures reported here, the infusorians were observed to absorb readily starch in contrast to protein which remained in the nutrient medium, even after being kept in a thermostat for 24 hours. This would be indicative of protein being not completely utilized, not even in the case of the smallest amount (10 mg.) of protein introduced. Protein may also be supposed to be the nutrient medium for the bacterial flora which is, perhaps, another protein source for the infusorians. The dependence of the infusorian number on the kind of the fermentation process may be an indirect evidence of this. Such a definition is probable to be very insufficient and general; however, the direct effect of the fermentation products may not be omitted as the latter may considerably affect the propagation of the infusorians. The observations as were carried out on the cultures permitted to suppose that changes occurred following each transfer from gluten (the initial medium) onto a new medium differring in the sort of protein contained, and regulation of fermentation processes, favourable to the normal development of infusorians, occurred. These observations proved that some other bacterial flora developed, decisive of the change in the fermentation processes; at the same time, these phenomena might well be brought about by the occurrence of proper nutrient medium.

The opinions of Ferber 1928 or that of Mowry et Becker 1930 do not solve the problem of the digestion of vegetable proteins by the infusorians; yet they cast some light on the problem and supply some amount of important evidence.

Ferber 1928, who fed to sheep food of various nutritive value, showed the increase in infusorian population in the rumen to occur when the animals were fed fodder containing much protein or when the protein metabolism in the host was more intense as usual (e. g. during pregnancy or lactation). Another Ferber's opinion, namely that the host gains advantage from the digestion of the not easily assimilated vegetable proteins as were transformed into accessible animal proteins in the infusorian organism, permits to suppose the infusorians being able to utilize vegetable proteins.

Similar is the opinion of Mowry et Becker 1930 who held the size of the infusorian population in the goat to depend on the kind of food ingested by the host, the physiological condition of the latter (pregnancy, lactation) being the second factor causing the infusorian population to increase.

The infusorian population more than doubled (from 1301000 to 2695000) within 5 days in experiments in which the animals were added some gluten to their normal ration of hay and starch, as compared to the control group (from 388000 to 1469000 specimens within 10 days). This result does not only corroborate the opinion of the above authors, but also is a test, to some extent, of the correctness of the conclusions reported here. It ought to be mentioned, however, that both the opinion of Ferber or that of Mowry et Becker, on the one hand, and their findings, on the other (experiments in situ) may thus be compared with and apply to the results reported here with much reserve only taking into consideration different growth conditions created by the specific artificial surroundings of the infusorian culture.

The studies reffered to above do not define the part bacteria play in the process of vegetable protein digestion, the only mention of that question being the suppositions of Mowry et Becker 1930. They admit that the population increase of the infusorians may possibly depend on the development of the bacteria owing to the food occurring in the substratum. This opinion on the problem of protein digestion in the rumen of ruminant may be based on the fact that Liebetanz 1905, Ferber 1928, and Westphal 1935 confirmed bacteria in the endoplasmatic sack of the infusorians.

Sym et coll. 1939 confirmed the presence of proteolitic enzyme, probably of bacterial origin which, when absorbed, acted on the solid particles of the rumen contents according to the authors; this observation too points to the possible bacterial digestion of proteins.

Similar enzymic studies were carried out by Schlottke 1936 on extracts prepared of infusorians and rumen bacteria of cattle. The author confirmed infusorial proteinase, which he defined as tripsine, and a strong dipeptidase. The effect of the enzyme was considerably weaker in bacterial extract, thus enabling Schlottke to differentiate the enzymes mentioned. This kind of examinations supply very convincing evidence; still, the results obtained might not be generalized. The confirmation of proteolitic enzyme occurring in the extract of the most frequent protozoans could be indicative of all the rumen infusorians ability to decompose protein; however, no sufficient evidence is available so far to permit such a generalization. The investigations carried out on monocultures supply data to justify the division of functions among the infusorians depending on food. This view point is at variance with the opinion according to which the rumen protozoans are regarded as a whole as food requirements are concerned.

The studies by Sym and by Schlottke do not suply the answer to the question considered here, namely: whether the vegetable proteins are digested directly by the protozoans themselves or indirectly through bacteria. It follows from the results obtained by the above authors that both of these interpretations may be equally valid and possible.

Another important problem is the definition of reasons as caused the very

considerable increase in the culture of the gliadin and serradilla fed infusorians. In cultures grown on two different gliadin levels (10 and 20 mg.), the gradual decrease in the population size is indicative of unfavourable conditions for infusorian development. The addition of vegetable parts to the nutrient medium was thus confirmed to bear a favourable effect on the population size of the infusorians. The vitality of the protozoans improves very markedly, and their considerable vitality is indicative of the medium having been also properly selected which fact secured the survival of the cultures over the long period of investigations.

On several occasions, *Entodinium longinucleatum* were observed to snatch out fragments of serradilla. Such infusorians occurring sporadically only, the utilization of serradilla may be supposed rather insignificant, and the vegetable fragments to represent no appropriate food. This conclusion is in accordance with the findings of Ciemochowska-Mazik 1956 who proved that *Entodinium longinucleatum* did not develop normally in culture on nutrient medium consisting of green vegetable particles unless some starch was added. In cultures supplemented with serradilla, some regulation of fermentation process seems to occur; this, probably, would be induced by the development of appropriate bacteria which make nutrient medium similar to the typical natural conditions in the rumen.

The specificity in physiological requirements of the individual infusorian species, and the complex processes of the medium in which they can develop, represent major obstacles in answering the question of the intricated relations between the infusorians and the host.

The present study does not permit to draw any decisive conclusions, yet, the findings as compared with further experiments on bacteria-free cultures or with enzymatic studies on definite species, could cast more light on the question of digestion in the infusorians, especially on that of proteins.

The results obtained permit to confirm the components of the nutrient medium having been properly composed thus securing a relatively long period of the *Entodinium longinucleatum* development. Thanks to the proper composition of the conditions, the authoress was enabled to obtain large numbers of infusorians, the highest population size on the gliadin fraction with serradilla being 83 766 infusorians per 1 cc. The size of infusorian populations obtained show to the best advantage if compared with the 40 500 specimens per 1 ml. obtained by Ciemochowska-Mazik in the barley-starch-containing medium, and with the 2000 to 5000 specimens per 1 cc., on the average, obtained by Hungate in inorganic nutrient medium with some starch and ground wheat added, in his experiments on *Entodinium caudatum*.

Conclusions

1. Vegetable protein is utilized by *Entodinium longinucleatum* either directly or indirectly through the bacterial flora.

2. Of the three sorts of protein contained in the nutrient medium, gluten has proved of full value.

3. The lack of serradilla particles in the endoplasmatic sack seems to be indicative of this food component being not directly utilized by *Entodinium longinucleatum.*

4. The high proportion of infusorians in medium supplemented with serradilla would be indicative of this food component being indirectly utilized by *Entodinium longinucleatum.*

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STRESZCZENIE

Entodinium longinucleatum hodowano w kulturach in vitro w warunkach anaerobowych stosując jako podłoże sok żwacza, a jako pokarm białka pszenicy: gluten, gliadynę i glutenin. Dodatkowym składnikiem była skrobia jęczmienna i suszone liście seradeli. Miarą wyzyskania poszczególnych białek była ilość wymoczków w 1 cm^3 soku kultury kontrolowana co 24 godziny w ciągu całego okresu hodowli.

Otrzymane wyniki pozwalają wnioskować, że białka stosowane w hodowli mogą być wykorzystane bezpośrednio bądź też pośrednio przez florę bakteryjną, która, być może, uzupełnia również dietę *E. longinucleatum*. Spośród trzech stosowanych białek (z dodatkiem skrobi jęczmiennej) największą ilość wymoczków otrzymano na glutenie, a wynik ten zależny jest prawdopodobnie od składu ilościowego aminokwasów. Ponieważ w woreczku endoplazmatycznym wymoczków w bardzo rzadkich przypadkach znajdowano fragmenty liści seradeli, wnioskuje się, że ten składnik pokarmu nie jest wykorzystywany przez wymoczki. Natomiast największa liczba wymoczków (83 766 w 1 cm^3), jaką otrzymano w hodowli stosując jako pokarm gliadynę i seradelę, utwierdza w przeświadczeniu, że poważnym uzupełnieniem diety wymoczków jest flora bakteryjna jako pośrednie ogniwo, wiążące wymoczka *E. longinucleatum* z pokarmem i żywicielem.